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Research Note

Enzyme-Linked Immunomagnetic Electrochemical Detection of Live *Escherichia coli* O157:H7 in Apple Juice†

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ABSTRACT

We describe the application of enzyme-linked immunomagnetic electrochemistry (ELIME) for the rapid detection of *Escherichia coli* O157:H7 in buffered apple juice. The ELIME technique entails sandwiching bacterial analyte between antibody-coated magnetic beads and an alkaline phosphatase–conjugated antibody. The beads (with or without bound bacteria) were localized onto the surface of magnetized graphite ink electrodes in a multiwell plate format. The enzyme substrate, 1-naphthyl phosphate, was added, and conversion of substrate to an electroactive product was measured using electrochemical detection. With this technique, detection of whole, live *E. coli* O157:H7 bacterial cells was achieved with a minimum detectable level of ca. 5×10^3 cells per ml in Tris-buffered saline or buffered apple juice in an assay time of ca. 80 min. With adjustment of pH, the ELIME response for the bacteria in either sampling medium was similar, indicating that apple juice components did not contribute to any discernible sample matrix effects.

Rapid bacterial detection methods have been developed as alternatives to lengthy and laborious, yet selective and highly sensitive conventional culture techniques *(7, 8, 11).* Some of these methods have employed the selectivity of immunoassays and both the rapidity and sensitivity of electrochemistry for the detection of molecular analytes or bacteria *(4, 5, 10, 12).* In addition, several methods have combined the selectivity and potentially high surface area–mediated capture capability of immunomagnetic beads (IMB) with electrochemistry for the detection of molecular analytes or bacteria *(9, 13, 16, 17).*

In this study, we have applied enzyme-linked immunomagnetic electrochemistry (ELIME) to the rapid detection of whole *Escherichia coli* O157:H7 cells in either buffer or apple juice. Apple juice has been selected as a target sample matrix due to the past association of the pathogenic bacteria, *E. coli* O157:H7, with outbreaks of food poisoning and hemolytic uremic syndrome linked to the commodity *(1–3).* The ELIME methodology involved sandwiching of *E. coli* O157:H7 cells between IMB and alkaline phosphatase–conjugated antibody. The IMB (with or without bound bacteria) were localized onto graphite ink strip electrodes with the aid of permanent magnets. Enzyme substrate (1-naphthyl phosphate) then was added, and conversion to an electroactive product was quantified using Osteryoung square wave voltammetry.

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MATERIALS AND METHODS

Materials. Materials used in this research included alkaline phosphatase–conjugated goat anti–*E. coli* O157:H7 (anti–*E. coli* O157:H7 conjugate, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), *E. coli* O157:H7 B1409 (Centers for Disease Control and Prevention, Atlanta, Ga.), goat anti–*E. coli* O157 M-280 IMB, magnetic particle concentrator (Dynal Inc., Lake Success, N.Y.), graphite ink silk screen printed on Mylar (1-cm wide graphite ink strips separated by 3 mm on Mylar sheets measuring 31 by 38 cm, The Motson Co., Inc., Flourtown, Pa.), Alnico magnets (cylindrical 4.8-mm diameter by 25.4 mm, Edmund Scientific Co., Barrington, N.J.), 1-naphthyl phosphate (1-NP; disodium salt, 98%) and platinum wire (0.25-mm diameter, 99.99%, Aldrich, Milwaukee, Wis.), 1% Blocker casein (Pierce, Rockford, Ill.), brain heart infusion (Difco, Becton Dickinson, Sparks, Md.), and double coated plastic Tuck Carpet Installation Tape (Tesa Tape Inc., Charlotte, N.C.). Other chemicals used were of reagent grade. Pasteurized apple juice (''100% juice reconstituted from concentrate'') that contained no added preservatives or sweeteners was purchased from a local vendor.

Apparatus. All reactions with shaking were performed on a Vortex-Genie 2 (Scientific Industries, Bohemia, N.Y.). Bacteria samples were counted on a Petroff-Hausser bacteria counting chamber (Thomas Scientific, Swedesboro, N.J.). Electrochemistry of samples was performed in a custom-built multiwell electrode/magnet assembly that was constructed of polymethyl methacrylate blocks, Alnico magnets, double-sided tape, and graphite ink strip electrodes as previously described *(9).* All electrochemical measurements were obtained with a BAS CV-50W electrochemical analyzer (Bioanalytical Systems, Inc., West Lafayette, Ind.) with accompanying BAS 50W software (version 2.0). An Ag/AgCl reference electrode (0.6 cm by approximately 7 cm, Vycor tipped, Bioanalytical Systems, Inc.), wrapped with a platinum wire that

served as a counter electrode, was inserted into the tested solutions during electrochemical measurements.

Growth and enumeration of *E. coli* O157:H7. A loopful of *E. coli* O157:H7 cells collected from a slant was inoculated into 25 ml of brain heart infusion broth and incubated at 37°C for 18 h. A portion of the cells was serially diluted in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, pH 7.6) to 1:10 and 1:100 dilutions. Bacteria in an aliquot (6 μl) of the 1:100 dilution were enumerated using only the center grid (0.2 by 0.2 mm) of a Petroff-Hausser counting chamber. Enumeration was repeated three times using additional 6 μl aliquots of the 1:100 dilution, and a mean (± standard deviation) was determined for the four obtained values. The 1:100 dilution was used for further serial dilution in the preparation of samples for the generation of calibration curves. The subsequent error (standard deviation) in the determined bacteria concentration (mean value for the 1:100 dilution) was propagated taking into account an estimated error of 5% for volumetric measurements.

Purification of 1-NP. To reduce electrochemical background response associated with oxidation of 1-napthol possibly present in 1-NP, the 1-NP was purified using the following procedure: 50 mg of 1-NP (gray crystals) was placed into a glass tube and dissolved in 0.5 ml of methanol (high-performance liquid chromatography grade); 50 mg of charcoal was added along with an additional 0.5 ml of methanol; the mixture was warmed to approximately 37°C for 10 min followed by gravity filtration through filter paper (qualitative 2; Whatman Limited, England) into a glass vial; 2 ml of warmed methanol was used to rinse the test tube and filter paper; nitrogen gas was passed over the filtrate until the solvent was evaporated, and the walls of the vial were scraped with a metal spatula to retain the white 1-NP powder that was stored at −20°C until used.

ELIME detection of bacteria. *E. coli* O157:H7 bacteria contained in a 1:100 dilution (in TBS) of stock were further serially diluted in either TBS or unbuffered apple juice. Where indicated, the pH of the apple juice samples was then adjusted to 7.6 using 1 M Tris (76 μl per ml of apple juice). With reaction volumes suggested by Dynal Inc., 20 μl of IMB were placed in 1.5-ml polypropylene microcentrifuge tubes, 1 ml of bacteria (in either TBS or apple juice) was added, and the mixture was incubated by shaking (minimal agitation sufficient to prevent settling of the IMB) for 30 min. The tubes were placed into the magnetic particle concentrator for 3 min in order to trap the IMB (a portion containing bound bacteria when present) against the walls of the tubes, and the liquid was removed by aspiration. (During IMB concentration, the particle concentrator was gently inverted several times to retain any IMB located in the cap of the tubes and to focus the IMB into a single spot.) The IMB were resuspended by gentle vortexing with 1 ml of anti–*E. coli* O157:H7 (diluted 1:500 in 1% Blocker casein) for 30 min. The IMB again were separated using the magnetic particle concentrator for 3 min, and the liquid was removed. The IMB then were washed and resuspended twice with 1 ml of TBS followed by magnetic separation for 3 min in the magnetic particle concentrator and removal of the liquid after each wash. Finally, the IMB were resuspended with 0.2 ml of TBS.

For electrochemical analysis, 200 μl of IMB (with or without bound bacteria) were added to the solution holding block of a multiwell electrode/magnet. The beads were magnetically trapped against the electrodes for 2 min, and the liquid was removed by aspiration. With the magnetic field applied, 200 μl of 1-NP (2.7 mM in 0.2 M Tris, pH 9.6) was added to the well and allowed to react for 3 min. Production of electroactive 1-napthol was measured using Osteryoung square wave voltammetry (50 to 500 mV, 25 mV sweep width amplitude, 5 Hz frequency, 4 mV step potential, 10^{-5} A/V sensitivity) and the peak current was determined by drawing a tangent line across the base of the peak using the BAS 50W software. The electrochemical detection of immunomagnetically captured bacteria is schematically represented in Figure 1.

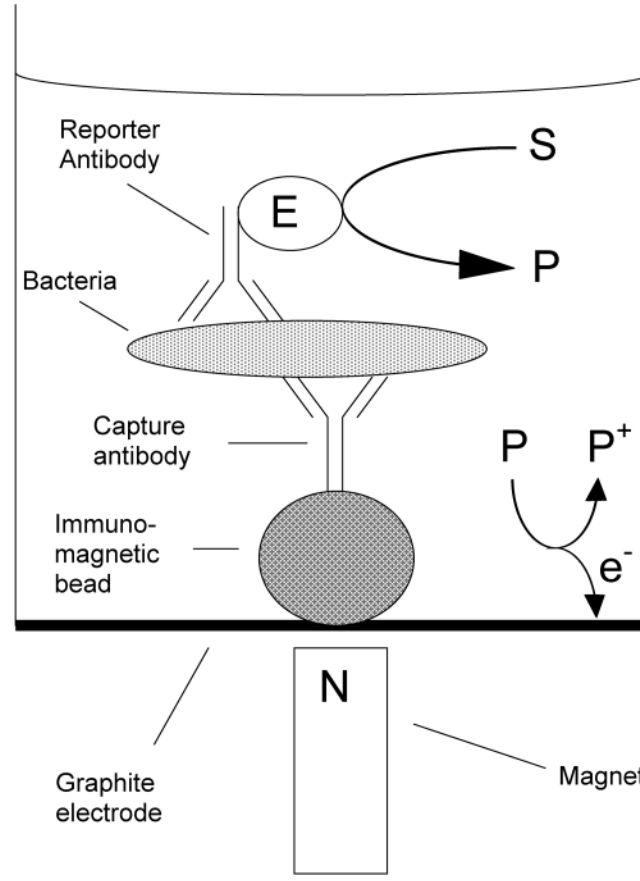

FIGURE 1. *Schematic representation of the enzyme-linked immunomagnetic electrochemical (ELIME) assay. Multi-ply immunogenic analyte (bacteria, for example) is sandwiched between antibody-coated magnetic beads (IMB) and enzyme-labeled antibody conjugate (reporter antibody). IMB (with or without bound bacteria) are magnetically trapped onto the electrode surface and exposed to substrate, and electroactive product is electrochemically detected. E, enzyme; S, substrate; P, product; and P^+, oxidized product.*

RESULTS AND DISCUSSION

The ELIME procedure entails the capture of multiantigenic analyte (e.g., bacteria) by IMB, labeling with enzyme (e.g., alkaline phosphatase)–conjugated antibody in a sandwich immunoassay format, magnetic concentration of the sandwiched bacteria at an electrode, and subsequent electrochemical analysis after a brief reaction with an electroactive enzyme substrate (Fig. 1). In a preliminary experiment, ELIME was used for the detection of live *E. coli* O157:H7 inoculated into untreated apple juice as compared to buffer (TBS). The results, portrayed as the mean (± standard deviation) electrochemical response (current) of triplicate measurements for one experiment, are displayed

TABLE 1. *Enzyme-linked immunomagnetic electrochemical (ELIME) detection of live* E. coli *O157:H7 (bacteria) in pristine, Tris-buffered saline (TBS) versus unbuffered apple juice*[a]

Sample	No. of bacteria	ELIME response (current; μA)	Standard deviation (current; μA)
TBS buffer blank	0	0.05819	0.02340
TBS buffer	$<4 \times 10^5$/ml	10.36	3.040
Apple juice blank	0	0.04308	0.02480
Apple juice	$\sim 4 \times 10^5$/ml	3.812	1.120

[a] Bacteria ($\sim 4 \times 10^5$/ml) were inoculated into TBS or untreated apple juice and analyzed using ELIME. The table values represent average responses of triplicate measurements ± standard deviation.

in Table 1. The presence of weak acids (ascorbic, citric, malic) in the apple juice accounted for an initial pH of approximately 3.6 for the juice. The dramatically reduced (approximately 60% lower) ELIME response observed for bacteria inoculated into untreated apple juice relative to TBS was presumed to be due to relatively poor recovery of the bacteria by the IMB, hence, pH inhibition of immunological reaction.

Results dictated that the pH of apple juice samples should be adjusted to that of the TBS (pH 7.6) prior to reaction with IMB. Based upon the titration of apple juice with standardized NaOH, at least 58 meq of base would have to be added to neutralize the weak acids contained therein, and a relatively small, additional amount of base would be required to further raise the pH to 7.6. Tris base solely was employed to maintain the same base system as used in the comparative TBS and since the resultant ionic strength would be similarly sufficient in prevention of potential bacterial cell lysis by osmotic pressure. Interpolation of the titration results (data not shown) indicated that 76 μl of 1 M Tris was required to adjust 1 ml of apple juice to pH 7.6.

Further experiments were performed to compare ELIME calibration curves for live *E. coli* O157:H7 in buffer (TBS) versus buffered apple juice. The experimental results, representing the ELIME detection of whole, live *E. coli* O157:H7 cells in TBS or Tris-buffered apple juice is displayed in Figure 2. Figure 2 displays the electrochemical response versus the number of *E. coli* O157:H7 bacteria per milliliter tested. ELIME detection of the bacteria exhibited very similar responses (sensitivity and slopes) for either of the sample matrices. The error displayed for the electrochemical response (current) was the standard deviation from the mean for measurements in triplicate for one experiment, whereas the error in the bacteria concentrations was derived from combining the error associated with enumeration of the initially diluted bacteria solution with estimated volumetric errors of 5% propagated over the range of serial dilutions.

The total assay time, based on a single sample, was approximately 80 min for ELIME. However, since the samples are concurrently reacted in a multiwell format, only approximately 5 min is required for the analysis of additional samples. Conservatively estimating the limit of detection to be 10 times the standard deviation of the blank response added to the blank response, substitution in the respective regression lines yielded limits of detection for *E. coli* O157:H7 to have been 3,300 cells per ml (in TBS) and 1,500 cells per ml (in buffered apple juice). The performance of ELIME for the detection of *E. coli* O157:H7 in apple juice favorably compares with other reported methods: 1,000 to 2,000 cells per ml in <1 h *(17)* and approximately 10^3 to 10^4 cells per ml in <1 h *(15)*. However, a risk assessment study *(6)* of the prevalence of the pathogen in apple cider indicates very low levels of potential contamination (i.e., 3 to 9 CFU/1,000 apples). Hence, as has been shown in past reports *(14, 18)*, enrichment culture must be employed prior to detection with select biosensors

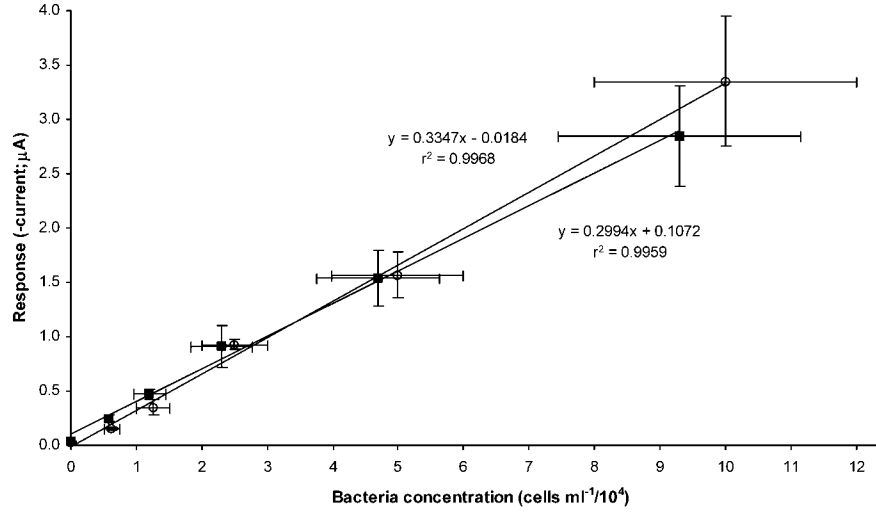

FIGURE 2. *ELIME detection of whole, live* E. coli *O157:H7 cells (bacteria).* E. coli *O157:H7 cells were serially diluted in either TBS or apple juice (pH adjusted with 1 M Tris as described in "Materials and Methods"), aliquots (1 ml) were immunomagnetically captured with anti–*E. coli *O157:H7 IMB, reacted with alkaline phosphatase–labeled anti–*E. coli *O157:H7 antibody conjugate, and detected electrochemically as previously described. The plot displays the electrochemical (current) responses for the varying concentrations of bacteria (○, in TBS; ■, in pH adjusted apple juice) that were tested.*

and biosensor-based methods (including ELIME in its current state of development) in order to achieve superior detection limits of ca. 2 to 10 CFU/ml or less.

REFERENCES

1. Anonymous. 1996. Outbreak of *Escherichia coli* O157:H7 infections associated with drinking unpasteurized commercial apple juice—Br. Columbia, Calif., Colo., and Washington, October 1996. *Morb. Mortal. Wkly. Rep.* 45:975.
2. Anonymous. 1997. Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider—Conn. and New York, October 1996. *Morb. Mortal. Wkly. Rep.* 46:4–8.
3. Besser, R. E., S. M. Lett, J. T. Weber, M. P. Doyle, T. J. Barrett, J. G. Wells, and P. M. Griffin. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *JAMA* 269:2217–2220.
4. Brewster, J. D., A. G. Gehring, R. S. Mazenko, L. J. Van Houten, and C. J. Crawford. 1996. Immunoelectrochemical assays for bacteria: use of epifluorescence microscopy and rapid-scan electrochemical techniques in development of an assay for *Salmonella. Anal. Chem.* 68:4153–4159.
5. Brooks, J. L., B. Mirhabibollahi, and R. G. Kroll. 1992. Experimental enzyme-linked amperometric immunosensors for the detection of salmonellas in foods. *J. Appl. Bacteriol.* 73:189–196.
6. Duffy, S., and D. Schaffner. 2002. Monte Carlo simulation of the risk of contamination of apples with *Escherichia coli* O157:H7. *Int. J. Food Microbiol.* 78:245–255.
7. Feng, P. 1992. Commercial assay systems for detecting food-borne *Salmonella:* a review. *J. Food Prot.* 55:927–934.
8. Fung, D. Y. C. 1995. What's needed in rapid detection of foodborne pathogens. *Food Technol.* 49:64–67.
9. Gehring, A., C. Crawford, R. Mazenko, L. Van Houten, and J. Brewster. 1996. Enzyme-linked immunomagnetic electrochemical detection of *Salmonella typhimurium. J. Immunol. Methods.* 195:15–25.
10. Hadas, E., L. Soussan, I. Rosen-Margalit, A. Farkash, and J. Rishpon. 1992. A rapid and sensitive heterogeneous immunoelectrochemical assay using disposable electrodes. *J. Immunoassay* 13:231–252.
11. Hartman, P. A., B. Swaminathan, M. S. Curiale, R. Firstenberg-Eden, A. N. Sharpe, N. A. Cox, D. Y. C. Fung, and M. C. Goldschmidt. 1992. Rapid methods and automation in food microbiology, p. 665–746. *In* C. Vanderzant and D. F. Splittstoesser (ed.), Compendium of methods for the microbiological examination of foods, 3rd ed. American Public Health Association, Washington, D.C.
12. Jenkins, S. H., W. R. Heineman, and H. B. Halsall. 1988. Extending the detection limit of solid-phase electrochemical enzyme immunoassay to the attomole level. *Anal. Biochem.* 168:292–299.
13. Robinson, G. A., H. A. Hill, R. D. Philo, J. M. Gear, S. J. Rattle, and G. C. Forrest. 1985. Bioelectrochemical enzyme immunoassay of human choriogonadotropin with magnetic electrodes. *Clin. Chem.* 31:1449–1452.
14. Seo, K. H., R. E. Brackett, and J. F. Frank. 1998. Rapid detection of *Escherichia coli* O157:H7 using immuno-magnetic flow cytometry in ground beef, apple juice, and milk. *Int. J. Food Microbiol.* 44:115–123.
15. Tortorello, M. L., and S. M. Gendel. 1993. Fluorescent antibodies applied to direct epifluorescent filter technique for microscopic enumeration of *Escherichia coli* O157:H7 in milk and juice. *J. Food Prot.* 56:672–677.
16. Weetall, H. H., and T. Hotaling. A simple, inexpensive, disposable electrochemical sensor for clinical and immuno-assay. *Biosensors.* 3:57–63.
17. Yu, H., and J. Bruno. 1996. Immunomagnetic-electrochemiluminescent detection of *Escherichia coli* O157 and *Salmonella typhimurium* in foods and environmental water samples. *Appl. Environ. Microbiol.* 62:587–592.
18. Yu, L., S. Reed, P. Tarkkinen, and S. Tu. 2003. Detection of *Escherichia coli* O157:H7 from food by a microplate sandwich immunoassay using time-resolved fluorometry. *J. Rapid Methods Automation Microbiol.* 11:133–143.